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Screening a series of sialyltransferases for possible BACE1 substrates

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Abstract Deposition of amyloid β-peptide (Aβ) and neurofibrillary tangles in the brain are hallmarks of Alzheimer's disease (AD) pathogenesis. BACE1, a membrane-bound aspartic protease that cleaves amyloid precursor protein (APP) to produce Aβ, has been implicated in triggering the pathogenesis of the disease. We previously reported that BACE1 also cleaved α2,6-sialyltransferase (ST6Gal I) in the Golgi apparatus and induced its secretion from the cell. Since most glycosyltransferases show Golgi localization and many of these are cleaved and secreted from the cell, we hypothesized that other glycosyltransferases may also be BACE1 substrates. Here, we focused on a series of sialyltransferases as candidates for BACE1 substrates. We found that BACE1 cleaved polysialyltransferase ST8Sia IV (PST) *in vitro*. We further found that BACE1 overexpression in COS cells enhanced the secretion of ST3Gal I, II, III and IV, although these sialyltransferases were not cleaved by BACE1 *in vitro*. These results suggest that BACE1 expression affects glycosylation not only by directly cleaving glycosyltransferases but also by modifying the secretion of glycosyltransferases via some other mechanisms.

Keywords BACE1 · Alzheimer's disease · Sialyltransferase · ST6Gal I, PST

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative dementia-inducing disorder. It is generally accepted that accumulation of amyloid β-peptide (Aβ peptide) in the brain is a crucial process for the pathogenesis of AD [8]. During the initiation of Aβ formation, amyloid precursor protein (APP) is cleaved by β-secretase and the residual membrane-bound COOH-terminal fragment is further cleaved by γ-secretase, resulting in the production of pathogenic Aβ peptide [2,3,27]. Since there is a large body of evidence suggesting that Aβ plays a causal role in AD pathophysiology, inhibiting Aβ generation and/or stimulating Aβ degradation are the most prominent strategies for AD treatment. Since γ-secretase also cleaves other biologically important molecules, such as notch [20,23,31], it is important to design a γ-secretase inhibitor that is specific for cleavage of APP. BACE1 (beta amyloid-converting enzyme 1) is a recently identified membrane-bound aspartic protease that was found to have β-secretase activity [1,10,22,26,30]. We previously demonstrated that BACE1 cleaves not only APP but also α2,6-sialyltransferase (ST6Gal I) [12–14]. Recent studies have further reported that BACE1 cleaves platelet selectin glycoprotein ligand-1 (PSGL-1) [16], voltage-gated sodium channels [29] and low-density lipoprotein receptor-related protein (LRP) [28]. To investigate the potential therapeutics of BACE1 inhibitors, several groups established BACE1 knockout mice and reported that BACE1 appears to

be responsible for the major β-secretase activity *in vivo*. Regarding the phenotype of the BACE1-deficient mice, several groups reported the mice are healthy and have no prominent neurological abnormalities. In contrast, another group reported abnormal characteristics, such as neurochemical and behavioral changes, in BACE1-deficient mice [9]. In other lines of BACE1 knockout mice, significant numbers of the offspring died after birth and the surviving knockout mice showed hyperactive behavior [4]. Overall, the impact of knocking out BACE1 *in vivo* remains unclear, and more studies on the behavior of BACE1 knockout mice are required to fully understand the more subtle cognitive and behavioral consequences of BACE1 ablation.

Many glycosyltransferases, including ST6Gal I, are type II membrane proteins that are retained in the Golgi apparatus for oligosaccharide biosynthesis [19]. Some of these enzymes are subsequently cleaved by proteases, and secreted out of the cell. Indeed, many glycosyltransferases have been found as soluble forms in bodily fluids such as serum, colostrum and milk [6,11,21]. Our previous finding that BACE1 is involved in the cleavage and secretion of ST6Gal I represents the first identification of a protease that plays a role in the secretion of glycosyltransferases. Our expectation that other types of glycosyltransferases may also be BACE1 substrates, and that such cleavage may partially reflect the abnormalities of BACE1-deficient mice, prompted us to screen a series of sialyltransferases for possible BACE substrates. We have previously showed that ST6Gal I and BACE1 were co-localized in Golgi [14]. Sialyltransferases, transfer a sialic acid residue to the non-reducing terminus of glycan chains in the trans Golgi and trans Golgi network, and hence their subcellular localization would be similar to that of BACE1. In the present study, we applied two lines of a screening strategy to a series of sialyltransferases in order to (i) examine whether BACE1 overexpression in cells stimulates secretion of sialyltransferases, and (ii) investigate whether purified BACE1 cleaves sialyltransferases *in vitro*.

Experimental procedures

Materials

Tissue culture media and reagents, including Dulbecco's modified Eagle's medium (DMEM), and Lipofectin were purchased from Invitrogen (Carlsbad, CA). Protein A Sepharose Fast Flow was purchased from Pharmacia Biotech Inc. (Piscataway, NJ). Fugene 6 was purchased from Roche (Mannheim, Germany). Columns for DNA purification were obtained from Qiagen Inc. (Chatsworth, CA). Protein molecular weight standards were purchased from Bio-Rad (Richmond, CA). Protein concentrations were determined using BCA protein assay reagents (Pierce, Rockford, IL). All other chemicals were obtained from Sigma or Wako Chemicals (Osaka, Japan).

Expression plasmids

For transient transfection experiments, human ST3Gal I-, II-, III- and IV- and ST8Sia IV-FLAG-pSVL were constructed as described previously [14,15]. BACE1-Fc and protein A-sialyltransferase (ST) chimeric proteins were generated by inserting the luminal parts of each ST into the EcoRI and XhoI sites of pcDSA encoding the signal peptide plus IgG-binding domain of protein A [14].

Effects of BACE1 overexpression on the secretion of a series of sialyltransferases by COS cells

COS-7 cells were maintained in DMEM containing 10% fetal bovine serum. The cells were plated on 150-mm tissue culture dishes and grown in a 5% CO_2 incubator at 37°C until they reached 50–70% confluence. ST FLAG-pSVL was co-transfected with either human BACE1-pcDNA3.1 cDNA or control pcDNA vector alone by the Lipofectin or Fugene method using Opti-MEM I**.** After 48 h of culture, intact ST-FLAG in cell lysates (40 μg of proteins) and soluble secreted ST-FLAG pulled down from the media with M2-agarose (Sigma) were subjected to 4–20% gradient SDS-PAGE, and then transferred to a nitrocellulose membrane. Next, the membrane was sequentially incubated with an anti-FLAG polyclonal antibody (1:1000) and a horseradish peroxidase (HRP)-goat anti-rabbit IgG secondary antibody (MP Biomedicals, Irvine, CA) before visualization with a chemiluminescent substrate (Pierce). ST-FLAG proteins detected with the anti-FLAG antibody were quantified using a Luminoimage Analyzer LAS-1000 PLUS (Fuji, Tokyo, Japan).

In vitro BACE cleavage assay

BACE1-Fc protein (or protein A-ST-FLAG) was purified from 20 ml of culture media obtained from COS cells that transiently expressed the protein. BACE1-Fc and protein A-ST-FLAG were absorbed by 20 μl of protein A-Sepharose and IgG-Sepharose (50% suspension in PBS), respectively. The reaction mixture contained 50 mM sodium-acetate buffer (pH 4.5) and 1 μl of BACE-Fc or protein A-ST6Gal I preparation, as well as Complete protease inhibitors (Roche, (Mannheim, Germany), 10 μM pepstatin, 1 μM leupeptin, 1 mg/ml pepstatin and 2 μM amastatin; Roche) to inhibit possible contaminating proteases. The reaction mixtures were incubated at 37°C for 0 or 60 min with rotation. The reaction products were analyzed by SDS-PAGE and immunoblotting with an anti-FLAG monoclonal antibody (1:1000), followed by incubation with an HRP-goat anti-mouse IgG secondary antibody (Sigma).

Results and discussion

Screening strategies to identify BACE1 substrates

We previously reported that overexpression of BACE1 in COS cells caused a significant increase in ST6Gal I secretion compared with that in control cells [14]. Current information showing that BACE1 prefers hydrophobic amino acid such at positions P1 and P3, was not enough to predict BACE1 substrate [7,25]. To examine whether the secretion of other sialyltransferases is increased by BACE1 overexpression, we applied a similar strategy to a series of sialyltransferases (ST) with a FLAG tag at their COOH-terminus, as illustrated in (Fig. 1A). Each ST-FLAG protein was overexpressed with or without BACE1 in COS cells. Secreted soluble ST-FLAG in the culture media, which was pulled down with M2-agarose, and intact ST-FLAG in the cell lysates were quantitatively analyzed by immunostaining using an anti-FLAG polyclonal antibody. We previously established an *in vitro* BACE1 cleavage assay, in which a purified recombinant BACE1-Fc chimeric protein was incubated with a purified ST6Gal I chimeric protein that lacked the transmembrane and cytoplasmic domains and contained a signal peptide plus protein A instead [14]. Since we determined that BACE1-Fc cleaved ST6Gal I at exactly the same site as *in vivo*, we rationalized that this *in vitro* system would be useful for screening possible BACE1 substrates. We therefore fused a signal peptide plus protein A with the luminal part of each ST-FLAG. A purified protein A-ST chimeric protein was used as a substrate for BACE1-Fc *in vitro* (Fig. 1B).

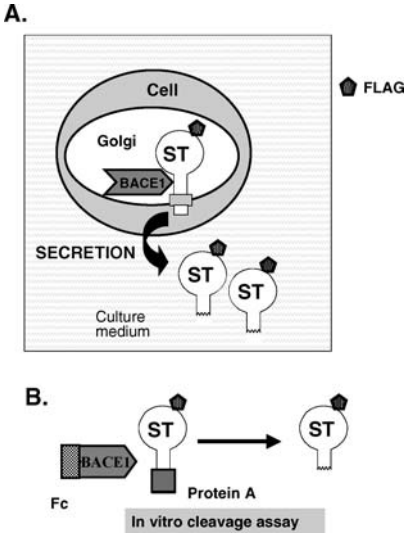

Fig. 1 Strategy for screening possible BACE1 substrates. **A**, Using cell-based screening, we investigated whether or not BACE1 overexpression in COS cells could enhance the secretion of a series of sialyltransferase (ST)-FLAG chimeric proteins. **B,** Using *in vitro* screening, we studied whether or not purified recombinant BACE1-Fc protein could cleave protein A-ST chimeric proteins

BACE1 overexpression enhances the secretion of ST3Gal enzymes

First, we focused on the ST3Gal family enzymes (ST3Gal I, II, III and IV), since some of these enzymes are found as soluble forms in extracellular fluids [5,18]. Each ST3Gal-FLAG protein was overexpressed in COS cells, and the secreted soluble forms of the ST3Gal-FLAG proteins were pulled down with M2-agarose and analyzed by immunostaining with an anti-FLAG polyclonal antibody. As shown in (Fig. 2), we detected soluble forms of all ST3Gal enzymes without co-transfection of BACE1, suggesting that an endogenous protease (or proteases) cleaves ST3Gal enzymes. The soluble forms of the ST3Gal II, III and IV enzymes secreted from the cells had higher molecular weights than their corresponding cellular forms. This may arise from differences in glycosylation, as proposed for *N*-acetylglucosaminyltransferase I [17]. More interestingly, the secretions of all the ST3Gal enzymes were significantly enhanced by BACE1 overexpression. Next, we used each protein A-ST3Gal-FLAG as a substrate for BACE1-Fc *in vitro*. Unexpectedly, we did not detect cleavage products for any of the ST3Gal enzymes (data not shown). These results suggest that BACE1 does not directly

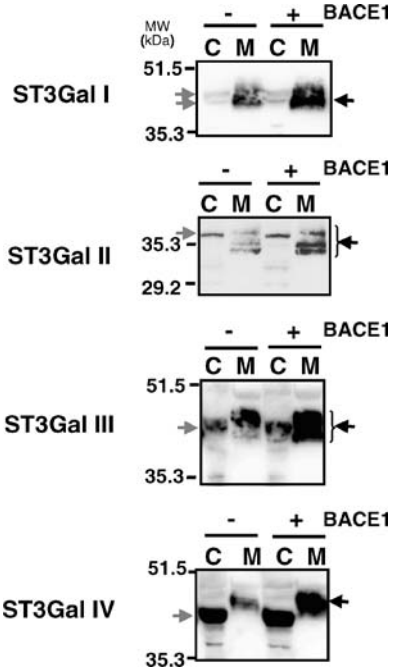

Fig. 2 Overexpression of BACE1 enhances the secretion of ST3Gal enzymes. ST3Gal enzyme (ST3Gal I, II, III, or IV)-FLAG-pSVL was co-transfected into COS cells together with BACE1myc-pcDNA or an empty vector. After 48 h of culture, the membrane-bound forms of the ST3Gal proteins in the cell lysates (80 μg of proteins) and the soluble ST3Gal-FLAG proteins, which were pulled down from the media with M2-agarose, were analyzed by immunostaining with an anti-FLAG polyclonal antibody. The membrane-bound forms of ST-FLAG in the cell lysates (C) and soluble secreted forms of ST-FLAG in the media (M) are indicated by gray and black arrows, respectively

cleave ST3Gal proteins, and that BACE1 overexpression indirectly affects ST3Gal secretion via another mechanism. One possible explanation is that BACE1 may activate a protease(s) that is responsible for the cleavage and secretion of ST3Gal proteins. Alternatively, BACE1 could inactivate the machinery for retention of ST3Gal proteins in the Golgi. Since ST3Gal I and II act on O-linked glycan chains, while ST3Gal III and IV prefer N-linked glycan chains [24], we are currently analyzing whether BACE1 affects the α2,3-sialylation of O-linked and/or N-linked glycan chains of cellular glycoproteins *in vivo*.

BACE1 cleaves ST8Sia IV *in vitro*

As mentioned above, we developed an *in vitro* assay system in which a series of protein A-ST-FLAG chimeric proteins were incubated with purified BACE1-Fc, and the reaction products were analyzed by immunostaining with an anti-FLAG antibody. As shown in (Fig. 3), we found that ST8Sia IV (PST) was cleaved by BACE1. The molecular weight of cleaved ST8Sia IV was similar to that of ST6Gal I after cleavage by BACE1. Less than 10% of ST8Sia IV was cleaved by BACE1, compared to more than 60% of ST6Gal I, under the same incubation conditions, indicating that ST8Sia IV is an inefficient substrate compared with ST6Gal I. Next, we overexpressed ST8Sia IV in COS cells with or without BACE1. In this case, however, the level of soluble secreted ST8Gal I was not increased in the presence of BACE1 (Fig. 4). Therefore, it remains unclear whether the observed cleavage of ST8Sia IV by BACE1 *in vitro* is an artifact or not.

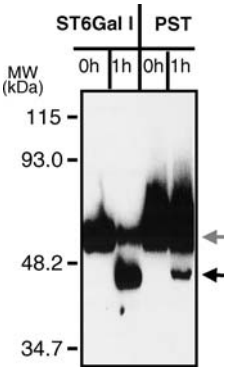

Fig. 3 BACE1 cleaves protein A-ST8Sia IV-FLAG *in vitro.* Recombinant BACE1-Fc protein was incubated with protein A-ST8Sia IV-FLAG or protein A-ST6Gal I-FLAG for 1 h. The reaction products were analyzed by SDS-PAGE, followed by immunostaining with an anti-FLAG polyclonal antibody. Gray and black arrows indicate the substrates and the cleavage products, respectively

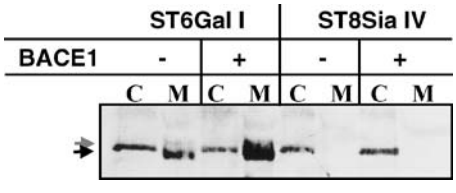

Fig. 4 ST8Sia IV secretion was not increased by BACE1 expression. ST8Sia IV-FLAG-pSVL was co-transfected into COS cells with BACE1-pcDNA or an empty vector. After 48 h of culture, ST8Sia IV-FLAG in the cell lysates (C, 30 μg of proteins) and soluble ST8Sia IV-FLAG, which was pulled down with M2-agarose from the media (M), were analyzed by immunostaining with an anti-FLAG polyclonal antibody. The secretion of ST6Gal I was evaluated as a control. The membrane-bound forms in the cell lysates (C) and soluble secreted forms in the media (M) are indicated by gray and black arrows, respectively

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